The Biological Activity of α-Mangostin, a Larvicidal Botanic Mosquito Sterol Carrier Protein-2 Inhibitor

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ABSTRACT α-Mangostin derived from mangosteen was identified as a mosquito sterol carrier protein-2 inhibitor via high throughput insecticide screening. α-Mangostin was tested for its larvicidal activity against third instar larvae of six mosquito species, and the median lethal concentration values range from 0.84 to 2.90 ppm. The residual larvicidal activity of α-mangostin was examined under semifield conditions. The results indicated that α-mangostin was photolytic with a half-life of 53 min in water under full sunlight exposure. The effect of α-mangostin on activities of major detoxification enzymes such as P450, glutathione S-transferase, and esterase was investigated. The results showed that α-mangostin significantly elevated activities of P450 and glutathione S-transferase in larvae, whereas it suppressed esterase activity. Toxicity of α-mangostin against young rats was studied, and there was no detectable adverse effect at dosages as high as 80 mg/kg. This is the first multifaceted study of the biological activity of α-mangostin in mosquitoes. The results suggest that α-mangostin may be a lead compound for the development of a new organically based mosquito larvicide.

KEY WORDS sterol carrier protein-2, larvicide, mangostin, detoxification enzymes, mosquitoes

David Fairchild described mangosteen (*Garcinia mangostana* L.) as the "queen of tropical fruit" in his 1930 book, *Exploring for Plants* (Fairchild 1930). According to Fairchild's account, the juicy, sweet-tasting fruit drove Queen Victoria to offer 100£ to anyone who could bring her one mangosteen. This feat would have been quite challenging in the 19th century, considering *G. mangostana* is a tropical evergreen that is mainly found in India, Myanmar, Sri Lanka, and Thailand (Jung et al. 2006). Almost a decade after Fairchild's account, the mangosteen received much scientific attention because of its xanthone-rich pericarp. Xanthones are heterocyclic compounds and are biologically active in numerous pathways (Fotie and Bohle 2006). Studies have found that xanthone derivatives from the pericarp of a mangosteen possess antiplasmodial (Mahabusarakam et al. 2006), anticancer (Nakagawa et al. 2007), antimicrobial (Mahabusarakam et al. 1986), and antioxidant (Jung et al. 2006) properties.

α-Mangostin is a mangosteen-derived xanthone that was identified during a high throughput insecticide screen as a mosquito sterol carrier protein-2 (SCP-2) inhibitor (SCPI) (Kim et al. 2005). SCPIs are a novel class of insecticides that target the SCP-2, which is partially responsible for intracellular cholesterol transport in insects (Larson et al. 2008, Lan and Massey 2004, Blitzer et al. 2005, Kim et al. 2005, Dyer et al. 2008). Cholesterol trafficking is essential for insects because they are unable to synthesize cholesterol de novo, and, as a result, insects rely on dietary sources of cholesterol (Clark and Bloch 1959). Therefore, the inhibition of cholesterol uptake and transport has consequential effects on insects. It has been demonstrated that SCPIs are toxic to mosquito and *Manduca sexta* larvae (Larson et al. 2008, Kim et al. 2005). Interestingly, SCP-2 is not critical for survival in vertebrates (Kannenberg et al. 1999, Fuchs et al. 2001). Moreover, α-mangostin has very low mammalian toxicity (Sornprasit et al. 1987). The novel mode of action and low mammalian toxicity combined with the fact that α-mangostin is extracted from the nonedible pericarp of the mangosteen fruit suggest that it might be developed as a promising organic insecticide.

Previous studies have found that α-mangostin has insecticidal properties against dipteran, coleopteran, and hemipteran pests. Whereas Ee et al. (2006) isolated α-mangostin and determined the 24-h median lethal concentration (LC_{50}) to be 19.4 μgml^{-1} against third instar *Aedes aegypti* L., the mode of action of the toxicological effects of α-mangostin against this mosquito species was not proposed. However, work performed in Thailand with the rice weevil (*Sitophilus oryzae* L.) and the brown plant hopper (*Nilaparvata lugens* Stal.) suggests that α-mangostin inhibits esterase, acetylcholinesterase, and glutathione S-trans-

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ferase (GST) activities (Bullangpoti et al. 2004, 2006). In this study, we evaluated α-mangostin as a SCPI for mosquito control. α-Mangostin was tested against six species of mosquito larvae and one adult species to determine the relative toxicity. The persistence of biological activity of α-mangostin in sunlight was determined in a semifield trial. We also revisited the report that α-mangostin possesses enzymatic inhibitory properties using a mosquito model.

Materials and Methods

Chemicals. All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), unless otherwise specified. All laboratory bioassays used a 50 mg ml^{-1} stock solution of α-mangostin (Gaia Chemical, Gaylordsville, CT) in which α-mangostin (98% purity) was dissolved in dimethyl sulfoxide (DMSO). For the semifield trials, α-mangostin (97% purity) was obtained from Indofine (Hillsborough, NJ) and a 50 mg ml^{-1} stock solution was prepared by dissolving it in DMSO. Technical grade temephos was provided by Clarke Mosquito Control (Schaumburg, IL). Temephos was dissolved in DMSO to prepare 1 mg ml^{-1} stock solution.

Mosquitoes. The Rockefeller strain of *Ae. aegypti* was reared in an incubator in Russell Laboratories located on the University of Wisconsin (Madison, WI) campus. Hatching was induced by transferring a piece of egg-covered filter paper to a 500-ml side arm flask filled with 250 ml of ddH$_2$0. The flask was placed under a vacuum for 20 min before the hatched larvae were transferred to a plastic tray containing 3000 ml of ddH$_2$0. The density was ≈1000 larvae/3000 ml ddH$_2$0. The larvae were fed Tetramin fish food and reared in an incubator maintained at 26°C, 70% RH, and with a photoperiod of 16 h:8 h (L:D).

Anopheles stephensi, Anopheles gambiae, and *Culex pipiens pipiens* larvae were obtained from Dr. Susan Paskewitz (University of Wisconsin, Madison, WI). Mosquitoes were maintained in an incubator at 26°C, 70–75% RH, and a photoperiod of 14 h:10 h (L:D). The eggs were then hatched in aluminum trays filled with deionized water. The larvae were provided a diet of VitaPro Plus fish food and brewer's yeast at a 2:1 ratio, respectively.

Ae. aegypti (Orlando strain), *Anopheles quadrimaculatus* Say, and *Culex quinquefasciatus* Say were reared in the insectary of the Mosquito and Fly Research Unit at Center for Medical and Veterinary Entomology (CMAVE), U.S. Department of Agriculture-Agricultural Research Service, according to the procedures described by Pridgeon et al. (2008). *Ae. aegypti* and *An. quadrimaculatus* have been established in the insectary since 1952 from Orlando, FL strains. *Cx. quinquefasciatus* has been established in the insectary since 1995 from a Gainesville, FL strain. Eggs were hatched in a flask with deionized water, left overnight, and transferred to a plastic tray containing distilled water. A powdered diet (2:1 pot belly pig chow:brewer's yeast) was added to each tray. Mosquitoes were reared in an environmental chamber at 22–30°C, 80% RH, and a photoperiod of 14 h:10 h (L:D).

Mammalian Toxicology Tests. Male Sprague Dawley rats, 20–21 d old (≈40–45 g), were obtained from Harlan (Indianapolis, IN) and housed individually in shoe box-type cages with wire lids. Rats were allowed free access to food (Product 8604, Teklad Laboratories Rodent Diet, Madison, WI) and water throughout the experiment, except when animals were fasted overnight before blood collection.

Animals were allowed to acclimate to their new environment for at least 3 d before initiation of the experiment. The α-mangostin solution for administration was prepared by dissolving it in DMSO at a concentration of 200 mg/ml. This was then diluted in DMSO to achieve the correct concentration for each animal. All animals, including controls, received solutions at a rate of 400 μl/kg body mass during each dosing.

Four different dosages were tested, as follows: 20 mg/kg ($n = 4$), 40 mg/kg ($n = 4$), 60 mg/kg ($n = 4$), and 80 mg/kg ($n = 4$). The compound was administered orally on a daily basis for consecutive 5 d, followed by a period of 2 d without treatment. This regime was carried out for consecutive 26 d. Control animals received equivalent volumes by body mass of the solvent for α-mangostin (DMSO; $n = 13$) or sterilized distilled water ($n = 8$). The water control was implemented to ensure that DMSO had no significant impact on the parameters being measured.

The physical condition of both treated and control animals was monitored daily. Animals were examined for signs of dehydration, lethargy, unresponsiveness, and stress (stress was noted by excessive porphyrin discharge from the eyes and nose). Animals were also weighed daily to check for signs of weight loss or to note unusual patterns in growth rate.

Blood samples were collected 5 d after administration of the compound began and every 7 d thereafter (i.e., days 5, 12, 19, and 26). Animals were anesthetized using isoflurane, and the blood was collected from the jugular vein. Blood was placed into heparinized tubes, and the plasma was extracted for analysis. The University of Wisconsin School of Veterinary Medicine (Madison, WI) analyzed the samples for serum levels of alanine aminotransferase (ALT), a biomarker of hepatocyte damage.

Seventy-Two-Hour Larvicidal Bioassays. Bioassays for the toxicity of α-mangostin were carried out against six species of mosquito larvae. All of the species were tested at the third instar. *Ae. aegypti* (Rockefeller), *An. stephensi,* and *Cx. pipiens pipiens* were tested in 60 ml of ddH$_2$0 using 60 larvae per container according to the methods described by Larson et al. (2008). The larvae used in these assays were maintained in an incubator at 26°C, 70% RH, and a photoperiod of 14 h:10 h (L:D). *Ae. aegypti* (Orlando), *An. quadrimaculatus,* and *Cx. pipiens quinquefasciatus* were tested at CMAVE, based on a modified World Health Organization method (WHO 2005). The bioassays performed at CMAVE used 100 ml of deionized water and 20 third instar larvae per container, and

were maintained at room temperature under constant lighting. In all assays, α-mangostin was serially diluted to six concentrations and fed a powdered diet every other day. DMSO equivalents were used as the control. The number of living and dead larvae was counted 72 h after the start of assay.

Continuous Versus 24-h Exposure to α-Mangostin to Mosquito Larvae. The effect of continuous exposure to α-mangostin versus 24-h exposure was investigated at CMAVE using *Ae. aegypti* (Orlando). Biological assays were performed, as previously described, using 100 ml of deionized water, 20 third instar larvae per container, and six concentrations of α-mangostin. However, in the 24-h treatment, larvae were removed from solutions of α-mangostin after 24 h, washed twice in deionized water, and then placed in 100 ml of deionized water for the remaining 48 h of the experiment. Controls consisting of equivalent concentrations of DMSO were set up for both the 24-h exposure treatment and continuous exposure treatment. Control larvae in the 24-h exposure treatment were removed from the DMSO solution and washed twice before being placed in 100 ml of deionized water. The number of living and dead larvae was counted 72 h after the start of the assay.

Mosquito Adult Bioassays. Adult bioassays were performed at CMAVE, as described by Pridgeon et al. (2008), with slight modifications. α-Mangostin was dissolved in acetone to make a 100 mg ml^{-1} stock solution before being serially diluted to six concentrations (3.13–100 mg ml^{-1}). These concentrations were selected after an initial screen determined that α-mangostin had biological activity in this range. Adult *Ae. aegypti* (Orlando) were used for the assays and were removed from a holding cage with an Insect Vac (BioQuip Products, Rancho Dominguez, CA) and placed on a chill table so the females could be separated from the males. Thirty female *Ae. aegypti* were placed in plastic cups covered with mesh screens for 24–48 h before the start of the bioassay. A cotton ball soaked in a 10% sucrose solution was placed on top of the mesh screen. The 5- to 7-d-old adult mosquitoes were anesthetized with CO_2 for ≈30 s before being transferred to a chill table (BioQuip Products, Rancho Dominquez, CA). A 700 series syringe and PB 600 repeating dispenser (Hamilton, Reno, NV) were used to apply 0.5 μl of α-mangostin solution topically to the dorsal thorax of the females. An equivalent amount of acetone was applied as a control. After the topical application, the mosquitoes were immediately transferred back into the plastic cups with a mesh top and provided with a cotton ball soaked in a 10% sucrose solution. Mortality was recorded 48 h after treatment.

Persistence of Biological Activity of α-Mangostin in Shade and Laboratory Conditions. The methods of Perez et al. (2007) were slightly modified to determine the persistence of α-mangostin in the shade and laboratory conditions. This experiment was performed at CMAVE from 28 July 2008 to 7 August 2008. A 40 μg ml^{-1} solution of α-mangostin was prepared in 5-gallon buckets lined with high-density molded polyethylene inserts (Associated Bag, Milwaukee, WI) with a total

volume of 5 L of deionized water. Containers in the shade treatment were carried outside between 9:00 and 10:00 a.m., covered with a plastic roof and an ultraviolet (UV)-resistant tarp, whereas the containers in the laboratory were kept in a room with no windows and maintained at 22°C. An initial sample (time 0) was taken before the buckets were carried to respective treatment locations. Each treatment included four containers that were independently sampled at time 0 (initial), 6 h, 12 h, 48 h, 120 h, and 240 h. Each sample was diluted to make four concentrations ranging from 2.5 to 40 μg ml^{-1}, and brought to a volume of 100 ml. Groups of 20 s to third instar *Ae. aegypti* larvae were added to each sample. Larvae were fed a powder diet every other day, and mortality was recorded at 72 h. A HOBO Pro Series Weatherproof Logger (Onset, Bourne, MA) was used to record the air and water temperatures of the shade sample. In the shade treatment, air and water temperature ranged from 23.5 to 40°C and 24 to 33.3°C, respectively. Four containers with an equivalent amount of DMSO were set in both shade and laboratory conditions, which served as the negative controls. The LC$_{50}$, heterogeneity factor (Hf), and 95% confidence intervals were calculated using Polo Plus logit probit software (LeOra Software, Petaluma, CA). The proportion of original toxicity was calculated as described in Perez et al. (2007).

Persistence of Biological Activity of α-Mangostin in Sunlight. Initial experiments found that α-mangostin exposed to sunlight for 6 h had reduced larvicidal activity. Because of α-mangostin's photosensitivity, the experimental design was modified to capture its short half-life. Four concentrations (2.5, 5, 10, and 20 μg ml^{-1}) were prepared in 20-L buckets lined with high-density molded polyethylene inserts (Associated Bag, Milwaukee, WI) with a total volume of 5 L. These experiments took place on 5 and 6 August at CMAVE. Sky cover was clear on both days (http://www.srh.noaa.gov/jax/f6/KGNV/F6_KGNV_AUG2008). The ambient temperature ranged from 20.6 to 33.9°C and 21.7 to 34.4°C on 5 and 6 August, respectively (http://www.srh.noaa.gov/jax/f6/KGNV/F6_KGNV_AUG2008). Containers were set outside between 9:00 and 10:00 a.m. and were kept under direct sunlight for the remainder of the experiment. Samples were taken at time 0 (initial), 45 min, 90 min, 180 min, and 360 min. The initial sample was taken before the containers were set outside to eliminate any effects of sunlight. Twenty second to third instar *Ae. aegypti* (Orlando) larvae were added directly to 100-ml samples taken at time 0 (initial), 45 min, 90 min, 180 min, and 360 min. On 5 August, the experiment was set up with two replicates, whereas it was set up with three replicates on 6 August. The number of living and dead larvae was counted after 72 h of exposure. Polo Plus probit and logit analysis software (LeOra Software, Petaluma, CA) was used to calculate the LC$_{50}$ and Hf. The proportion of initial toxicity was calculated as described in Perez et al. (2007).

Assays for Enzymatic Activities in Larvae. Twenty late third instar *Ae. aegypti* (Rockefeller) were pre-

exposed to 100 ml of water containing 2.5 μg ml^{-1} of α-mangostin for 24 h. This dose was chosen because it was predetermined as the 72-h LC$_{50}$. DMSO equivalents served as the negative control. The larvae were washed twice in ddH$_2$0 before being used for testing. After the 24-h induction period, the larvae molted to the fourth instar, and surviving larvae were used for biochemical tests. The mortality was <15% after 24-h exposure to 2.5 μg ml^{-1} of α-mangostin.

Cytochrome P450 Activity. Living larvae exposed to α-mangostin or DMSO were cleaned in ddH$_2$0 and dissected. Larvae were placed in 50 mM sodium phosphate buffer (pH 7.2) and chilled before dissection. The heads, last abdominal segment, and digestive system were removed from the larvae. Carcasses were then used for determining the cytochrome P450 activity. Cytochrome P450 assays were based on the measurement of ethoxycoumarin-O-de-ethylase activity in the body walls. The methods of Boyer et al. (2005) and De Sousa et al. (1995) were modified to determine the effect of α-mangostin on the cytochrome P450 activity in *Ae. aegypti* larvae. Black, round-bottom 96-well microplates (Catalog 3356, Corning Glass, Corning, NY) were filled with 100 μl of a 0.4 mM 7-ethoxycoumarin solution containing 50 mM sodium phosphate buffer (pH 7.2). Individual larval carcasses were placed in each well and incubated for 4 h at 30°C. The reaction was stopped with the addition of 50 μl of glycine buffer (1 mM, pH 10.4) and 50 μl of ethanol. Larval carcasses remained at the bottom of the well and were not removed before reading. Six wells containing 100 μl of phosphate buffer, 50 μl of glycine buffer, and 50 μl of ethanol served as the blank. The fluorescence of the reaction medium was measured from the top of the wells using a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT) with 400 nm excitation and 480 nm emission filters. The production of 7-hydroxycoumarin (7-OH) was expressed as μmol 7-OH/mg larvae/min.

Esterase Activity. The methods of Rodriguez et al. (2001) were modified to determine the effect of α-mangostin on the esterase activity of fourth instar *Ae. aegypti* (Rockefeller) larvae. Individual larvae were homogenized using a micropestle in a 1.5-ml Eppendorf microcentrifuge tube containing 200 μl of 50 mM sodium phosphate buffer (pH 7.5) before being centrifuged at 13,200 rpm for 15 min. A total of 20 μl of the supernatant was added to each well of a 96-well clear, radius edge, polystyrene microplate (Labsystems, Helsinki, Finland) before 160 μl of 0.5 mM β-naphthyl acetate was added. The reaction was incubated for 10 min at 30°C before it was stopped with the addition of 20 μl of Fast Blue B salt 0.3% containing 3.5% sodium dodecyl sulfate. A previous study using the Rockefeller strain of *Ae. aegypti* larvae under similar experimental conditions determined the saturation of concentration for β-naphthyl acetate to be 70 mM and the optimal reaction time to be 10 min (Rodriguez et al. 2001). The reaction was allowed to continue at room temperature for 10 min before the absorbance was read at 600 nm using a VersaMax

microplate reader (Molecular Devices, Sunnyvale, CA). This assay was performed in replicates of 12 and repeated three times ($n = 36$). Protein concentrations were determined using an albumin standard and bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). The esterase activity was expressed as μmol/mg protein/min substrate hydrolyzed.

GST Activity. GST activity samples were prepared according to the method of Rodriguez et al. (2001). Individual fourth instar larvae were homogenized in 200 μl of 50 mM sodium phosphate buffer (pH 7.2) before being centrifuged at 15,700 $\times$ g at 4°C for 15 min. The Sigma-Aldrich (St. Louis, MO) GST assay kit was used to measure the conjugation of the thiol group of glutathione to the 1-chloro-2, 4-dinotrobenzene (CDNB) substrate. A total of 20 μl of homogenate was added to each well before 180 μl of solution containing Dulbecco's phosphate buffer (Sigma-Aldrich, St. Louis, MO), glutathione reduced (4 mM), and CDNB (2 mM). Rodriguez et al. (2001) determined the saturation concentration of CDNB to be 50 mM and the optimum time for reading to be 3 min. The 96-well flat-bottom UV microplate (Corning Glass, Corning, NY) was immediately loaded onto a Synergy HT microplate reader (BioTek, Winooski, VT). After a 1-min lag time, the absorbance was read at 340 nm and the samples were read every 60 s for 3 min. Protein concentrations were determined using an albumin standard and bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). The activity of GST was expressed as μmol/mg protein/min substrate conjugated.

Synergistic Effect of α-Mangostin on Temephos Toxicity Against Mosquito Larvae. Third instar *Ae. aegypti* (Rockefeller) larvae were used to investigate potential synergistic effects of α-mangostin. Bioassays were performed in which temephos was serially diluted to six concentrations (0.008–0.047) with a 100 ml total volume and 20 larvae were transferred to each container. A 1 μgml^{-1} α-mangostin solution was added to an identical treatment of temephos. After 24 h, the number of living larvae was recorded. The control consisted of six identical plastic containers filled with a 1 μg ml^{-1} concentration of α-mangostin, which resulted in <5% mortality within 24 h.

Statistical Analysis. Probit analysis for all laboratory bioassays was carried out using StatPlus:mac software (AnalystSoft, Vancouver, British Columbia, Canada). The LC$_{50}$, LC$_{50}$ 95% confidence intervals, and 95% lethal concentration (LC$_{95}$) were calculated using this software. This software package does not calculate confidence intervals for the LC$_{95}$, and these values are not listed as a result. The Hf was calculated by dividing the χ^2 value by degrees of freedom. StatPlus:mac software (AnalystSoft, Vancouver, British Columbia, Canada) was also used to perform Student t tests to determine the significance of the enzyme activities between treatments. Polo Plus logit probit software (LeOra Software, Petaluma, CA) was used to calculate the LC$_{50}$, confidence interval, and Hf in the semifield portion of this study.

Table 1. Seventy-two-hour toxicity assay of α-mangostin against third instar larvae of six mosquito species

Species	n	LC_{50} μgml^{-1} (95% CL)	LC_{95} μgml^{-1}	Slope (SE)	Hf
Ae. aegypti (Rockefeller)	5	2.2 (1.9–2.6)	5.7	3.9 (0.4)	2.50
An. gambiae	4	2.9 (1.8–5.5)	12.2	2.7 (0.6)	5.05
An. quadrimaculatus	11	1.7 (1.5–1.9)	2.7	8.1 (1.4)	0.42
An. stephensi	6	1.9 (1.6–2.4)	8.1	2.6 (0.3)	1.46
Cx. pipiens pipiens	6	0.84 (0.8–0.9)	2.0	4.3 (0.2)	1.71
Cx. p. quinquefasciatus	8	1.6 (1.0–2.1)	4.2	3.8 (1.1)	3.93

Table 2. Continuous versus 24-h exposure on the 72-h toxicity in third instar *Ae. aegypti* larvae

Exposure time	n	LC_{50} μgml^{-1} (95% CL)	LC_{95} μgml^{-1}	Slope (SE)	Hf
Continuous	6	2.5 (2.3–2.7)	4.3	7.2 (0.8)	0.03
24 h	6	2.7 (2.3–3.3)	5.2	5.9 (0.9)	2.75

Third instar *Ae. aegypti* (Orlando) larvae were exposed to α-mangostin for 24 h and continuously. In the 24-h treatment, larvae were removed from α-mangostin solutions and washed twice before being transferred to ddH_2O for the remainder of the experiment (see *Materials and Methods*).

The body weights of the rats were compared for the different treatment groups at 7, 14, 21, and 28 d after compound administration began. Each time point was first analyzed using Bartlett's test to confirm that variance between the groups was uniform. A one-way analysis of variance was then conducted for each time point. If the analysis of variance showed significant differences between the groups ($P \leq 0.05$), a Dunnett's test was performed to determine which groups differed significantly from one another ($P \leq 0.05$).

To compare the rats' serum levels of ALT between the treatment groups, a repeated measure analysis using the mixed procedure in SAS (SAS Institute, Cary, NC) was performed. If a significant difference ($P \leq 0.05$) between the dosages was found, a pairwise test (with Bonferroni correction) was performed to determine which doses were significantly different from one another ($P \leq 0.05$).

Results and Discussion

Toxicity of α-Mangostin Against Larval Mosquitoes. Third instar larvae of six different mosquito species were used to evaluate the toxicity of α-mangostin. The results for the 72-h toxicity are shown in Table 1. *Cx. pipiens pipiens* and *Cx. pipiens quinquefasciatus* were the most susceptible to α-mangostin with an LC_{50} of 0.84 (0.81–0.89) μg ml^{-1} and 1.6 (1.0–2.1) μg ml^{-1}, respectively. *An. gambiae* was the least susceptible to α-mangostin with a 72-h LC_{50} of 2.9 (1.8–5.5) μg ml^{-1} and a LC_{95} of 12.2 μg ml^{-1}. SCPI-1, a synthetic SCPI, was evaluated previously against third instar larvae of *An. gambiae* and *Cx. pipiens* (Larson et al. 2008). In the previous study, the 72-h LC_{50} of SCPI-1 was 6.5 ± 3.0 μg ml^{-1} and 2.4 ± 0.8 μg ml^{-1} in *An. gambiae* and *Cx. pipiens*, respectively (converted from μM, molecular weight of SCPI-1 = 459.19 d). Compared with SCPI-1, α-mangostin is more effective against *An. gambiae* and *Cx. pipiens* by a factor of 2.2 and 2.9, respectively (SCP1–1 LC_{50}/α-mangostin LC_{50}). Interestingly, *An. gambiae* was the least susceptible to both SCPIs, whereas *Cx. pipiens* was the most susceptible. There are two possible explanations for the relative species-specific susceptibility to SCPIs. First, it is possible that there may be slight structural differences between the SCP-2 of *Cx. pipiens* and *An. gambiae*, which could result in differences in affinity for cholesterol and SCPIs (Larson et al. 2008). Second, exposure to SCPIs may induce higher levels of detoxification enzymes in *An. gambiae* compared with *Cx. pipiens*. Further studies are needed to test these possible mechanisms.

The 72-h LC_{50} in *Ae. aegypti* ranged from 2.2 to 2.5 μg ml^{-1} depending on the strain tested. The Rockefeller and Orlando strain of *Ae. aegypti* had similar susceptibility with LC_{50} values of 2.2 (1.92–2.6) (Table 1) and 2.5 (2.3–2.7) μgml^{-1} (Table 2), respectively. Our LC_{50} values were much lower than those found by Ee et al. (2006). They tested α-mangostin against third instar *Ae. aegypti* larvae and reported a 24-h LC_{50} of 19.1 μgml^{-1} (Ee et al. 2006). However, the 7.6-fold difference in LC_{50} value findings can be attributed to the differences in duration between the studies. The 24-h bioassays performed by Ee et al. (2006) were biased toward faster acting chemicals. Our previous study suggests that SCPIs are slow acting; therefore, evaluation of SCP-2 inhibitors should be performed over 72 h (Larson et al. 2008).

Continuous Versus 24-h Exposure to α-Mangostin. Although previous studies suggest that SCPIs are slow acting and should be evaluated for 72 h, it is not known whether continuous 72-h exposure to SCPIs is needed for effective control of mosquito larvae (Larson et al. 2008). In the current study, we exposed *Ae. aegypti* (Orlando) larvae to α-mangostin for 24 h and compared the toxicity to larvae that underwent continuous exposure to α-mangostin. It was found that *Ae. aegypti* larvae exposed to α-mangostin for 24 h had a 72-h LC_{50} of 2.7 (2.3–3.3) μgml^{-1}, whereas larvae exposed continuously had a 72-h LC_{50} of 2.5 (2.3–2.7) μg ml^{-1} (Table 2). The 95% confidence intervals of the two treatments overlap, which suggests that the difference between treatments is not significant at a 5% level. Thus, 24-h exposure to α-mangostin had detrimental effects on *Ae. aegypti* larvae that did not immediately result in mortality.

After 24-h exposure to α-mangostin (2.5 μgml^{-1}), large differences were also observed in the total soluble protein and mass of the body wall. The concentration of soluble protein from control larvae was 0.41 (0.36–0.45) mg ml^{-1} per larva, whereas the total soluble protein from larvae treated with α-mangostin was 0.17 (0.15–0.2) mg ml^{-1} per larva (Table 3). This is consistent with the average mass of larval body wall taken from 50 control larvae and 50 larvae exposed to 2.5 μgml^{-1} of α-mangostin. Larval body walls dissected from larvae that were exposed to DMSO and α-mangostin weighed 0.92 and 0.56 mg, respectively (Table 3). There was a greater relative difference between control and treated in amount of soluble protein (2.4-fold) compared with the difference of

Table 3. Soluble protein concentration and body-wall mass

	Mean (95% CL)	P value
Total soluble protein (mg/ml) ($n = 56$)		
Control	0.41 (0.36–0.45)	<0.001
α-Mangostin treated	0.17 (0.15–0.2)	
Average mass of body wall (mg) (average of 50)		
Control	0.92	NA
α-Mangostin treated	0.56	

Late third instar larvae of *Ae. aegypti* (Rockefeller strain) were exposed to 2.5 μgml^{-1} of α-mangostin for 24 h. Early fourth instar larvae were collected to determine soluble protein concentration and mass of fat bodies. Student *t* tests were used to determine P values. Average mass of body walls was calculated by weighing 50 carcasses and dividing their total weight by 50. Therefore, P values are not applicable to the average mass of body walls.

larval body wall masses between the control and treated (1.6-fold). Work performed by Kim et al. (personal communications) suggests that α-mangostin is a feeding deterrent on the Colorado potato beetle. It is possible that α-mangostin treatment led to decreased feeding and lipid metabolism in the larvae, which resulted in lowered body mass compared with the control. The Bradford method (Bradford 1976) of determining protein concentration cannot discriminate between protein from dietary sources in the midgut and protein from the insect body. α-Mangostin may be a feeding deterrent for mosquito larvae and could account for the relatively large differences in soluble protein. Nonetheless, the presence of α-mangostin drastically reduced the total soluble protein and average mass of larval bodies after only 24 h of exposure, whereas the 24-h mortality was <15%.

Toxicity of α-Mangostin Against Adult Mosquitoes. α-Mangostin was applied topically to 5- to 7-d-old adult *Ae. aegypti* (Orlando) to test its potential use as an adulticide. The results of this biological assay are summarized in Table 4. The 48-h 50% lethal dose (LD$_{50}$) was 2.2 (2.0–2.4) %wt:vol (mg compound/100 μl solvent), which is within the same order of magnitude as the LC$_{50}$ values found from bioassays performed against the rice weevil (*S. oryzae* L.) and the brown plant hopper (*N. lugens* Stal.) (Bullangpoti et al. 2004, 2006). Compared with the LD$_{50}$ values against mosquito larvae and commercially available insecticides, the LD$_{50}$ values for topically applied α-mangostin are several orders of magnitude higher. For instance, Pridgeon et al. (2008) evaluated 19 insecticides against adult *Ae. aegypti* using similar protocol to the one used in this study. It was found that *Ae. aegypti* were most susceptible to Fipronil with a LD$_{50}$ of 4.6×10^{-7} μg insecticide/mg mosquito and least susceptible to Bifenazate with a LD$_{50}$ of 1.5 μg insecticide/mg

Table 4. Forty-eight-hour toxicity assay of α-mangostin against 5- to 7-d-old adult *Ae. aegypti* (Orlando strain)

LD$_{50}$ (%wt:vol)	LD$_{90}$ (%wt:vol)	Slope (SE)	Hf
2.2 (2.0–2.4)	10.1	2.5 (0.1)	1.45

Table 5. Persistence of α-mangostin exposed to direct sunlight

Exposure to sunlight	LC$_{50}$ μgml^{-1} (95% CL)	Slope (SE)	Hf	Proportion of original toxicity remaining
0	4.9 (4.2–5.7)	3.2 (0.3)	1.73	11
45 min	10.3 (9.5–11.2)	4.6 (0.4)	1.05	0.48
90 min	21.8 (18.2–28.6)	2.7 (0.4)	1.40	0.22
Half-life = 52.8 min	Equation: $y = -0.0086x + 0.9544$			R^2 = 0.96

α-Mangostin was exposed to direct sunlight, and samples from various time points were tested against *Ae. aegypti* (Orlando) larvae. Proportion of original toxicity was calculated by dividing the 72-h LC$_{50}$ of each time point by the LC$_{50}$ corresponding to the initial sample (time 0). There was no mortality detected beyond 90-min time points, and LD$_{50}$ values could not be obtained after this time point.

mosquito (Pridgeon et al. 2008). Fourteen of the 19 compounds tested had LD$_{50}$ values $<10^{-2}$ μg insecticide/mg mosquito. When the LD$_{50}$ of α-mangostin is converted to the same units described in Pridgeon et al. (2008) (using the average weight of 7-d-old female *Ae. aegypti* = 2.85 mg and 0.5 μl of compound per female), the LD$_{50}$ of α-mangostin would be $\approx$4 μg insecticide/mg mosquito. Therefore, it can be concluded that α-mangostin has limited adulticidal properties compared with many commercial products.

There are several possible explanations for the limited activity of α-mangostin in adult *Ae. aegypti.* First, the extreme hydrophobicity of α-mangostin may hinder its ability to penetrate the cuticle. Second, α-mangostin may need to be ingested to be effective against insects because Aedes sterol carrier protein-2 (AeSCP-2) is highly expressed in the midgut (Krebs and Lan 2003). Third, SCPIs may not be effective against adult mosquitoes because AeSCP-2 is not highly expressed in the pupal and adult stages (Krebs and Lan 2003).

Persistence of Biological Activity of α-Mangostin in Semifield Conditions. When α-mangostin was exposed to direct sunlight, the initial (time 0), 45-min, and 90-min samples tested against third instar *Ae. aegypti* larvae (Orlando) produced LC$_{50}$ values of 4.9 (4.2–5.7), 10.3 (9.5–11.2), and 21.8 (18.2–28.6) μgml^{-1}, respectively (Table 5). LC$_{50}$ values from the initial time point (0 h) were approximately 2-fold higher than the 72-h LC$_{50}$ determined in laboratory assays (Table 2). These differences may be caused by the different containers used between studies. α-Mangostin is extremely hydrophobic, which might cause it to be more attracted to the liners used in the semifield assays than the plastic containers used in the laboratory assays. The LC$_{50}$ values for the 90-min sample were interpolated because two of the five samples did not produce at least 50% mortality. There was no mortality detected beyond 90-min time points, and LD$_{50}$ values could not be obtained after this time point. To determine the proportion of initial toxicity remaining, the LC$_{50}$ values for each time point were divided by the LC$_{50}$ value for the initial time point (0). These values were plotted over time. As predicted, the persistence had a negative linear response to sunlight. It was determined that α-mangostin has a half-life of

52.8 min with full sun exposure (Table 5). Relative to most commercially available pesticides, α-mangostin is very photosensitive. In the post "Silent Spring" era of pest management, environmental degradation of insecticides is a positive attribute (Carson 1962). However, insecticides must persist in the environment long enough for effective control. Formulation technology may also help photosensitive compounds overcome environmental degradation. For example, methoprene has a half-life of 30–40 h depending on the initial concentration (Schooley et al. 1975). Slow release formulations combined with UV-blocking additives allow some formulations of methoprene to remain biologically active in the environment for months. Thus, the photosensitivity of α-mangostin could potentially be corrected with existing formulation technology.

The persistence of α-mangostin was also evaluated in the laboratory and shade. Data were not shown for the shade and laboratory treatments, because α-mangostin precipitated out of solution in these treatments. α-Mangostin is practically insoluble in water (Merck Index 1989), which suggests that it dissociated with the carrier, DMSO. However, further studies are needed to determine the mechanism of the precipitation of α-mangostin stored in the shade and laboratory.

Biological Effects of α-Mangostin on Rats. Mammalian SCP-2s have low amino acid sequence identity to the mosquito SCP-2 (Krebs and Lan 2003); however, the three-dimensional structures show very high homology, with root mean square (rms) difference between 97 structurally equivalent amino acids only 1.15 Å (Dyer et al. 2003). Whether SCPIs such as α-mangostin would affect the function of vertebrate SCP-2 is unknown, although vertebrate SCP-2 seemed to be nonessential for mammalian survival (Kannenberg et al. 1999, Fuchs et al. 2001). To test the toxicity of α-mangostin in mammals, young male rats were fed varied dosages of α-mangostin for >4 wk, during which body weight and a biomarker for hepatocyte damage were measured. Survival rates for treated and control animals were 100%. During daily monitoring, no rats were found to be dehydrated, lethargic, unresponsive, or excessively stressed. The Bartlett test demonstrated that variance of body weight between the groups was uniform for each time point ($P \geq 0.47$). There was no significant difference in body weight between the groups at any of the given time points (day 7, $P = 0.709$; day 14, $P = 0.797$; day 21, $P = 0.808$; day 28, $P = 0.672$). The result of our toxicity assays in the young rats is consistent with the report on adult rats (Sornprasit et al. 1987).

Serum ALT levels differed significantly between time points ($P = 0.0002$), with levels of the enzyme decreasing in the bloodstream as the animals aged. However, there was no significant difference in serum ALT levels between groups ($P = 0.5007$) or when time point and dose were analyzed simultaneously ($P = 0.9463$), suggesting that α-mangostin did not result in hepatocyte damage that facilitated the release of ALT into the bloodstream. The results of this preliminary

toxicology study of α-mangostin in young rats suggest that α-mangostin exhibited little acute adverse effect in mammals, which is consistent with the observations in SCP-2 knockout studies that show that the vertebrate SCP-2 is not essential for survival (Kannenberg et al. 1999, Fuchs et al. 2001).

Enzymatic Responses of Fourth Instar Mosquito Larvae to α-Mangostin. In response to xenobiotics, insects must break down the chemical into soluble form before they can excrete it. To perform these tasks, insects often rely on three superfamily of enzymes: cytochrome P450 monooxygenases, esterases, and GST (Brogdon and McAllister 1998). Therefore, when exposed to a xenobiotic, enzyme activities are often induced to higher levels (Brattsten et al. 1986). The reasons for measuring cytochrome P450, esterase, and GST activities in the current study are 2-fold. First, these enzymatic activities were measured to investigate which enzymes are responsible for detoxifying α-mangostin. Second, enzyme activities were also measured to assess reports that α-mangostin inhibited detoxification of enzymatic activities. Studies in Thailand with the rice weevil (*S. oryzae* L.) and the brown plant hopper (*N. lugens* Stal.) suggest that α-mangostin inhibits esterase, acetylcholinesterase, carboxylesterase, and GST activities (Bullangpoti et al. 2004, 2006). On one hand, these findings are unexpected because α-mangostin is extremely hydrophobic and detoxification of enzyme activities is usually temporarily induced to higher activities by the introduction of foreign lipophilic compounds (Brattsten et al. 1986). However, it has been demonstrated that some plants have evolved to produce secondary metabolites with synergistic effects to enhance their chemical defense (Berenbaum and Neal 1985). Nonetheless, these claims need to be re-evaluated to determine whether α-mangostin has synergistic properties.

As expected, cytochrome P450 and GST activities increased significantly when *Ae. aegypti* (Rockefeller) larvae were exposed to 2.5 μg ml^{-1} of α-mangostin for 24 h, which suggests that these enzymes play a major role in its detoxification. The mean cytochrome P450 activity of the control and treatment were 6.4 ± 0.36 and 8.4 ± 0.6 μmol 7-OH/mg larvae/min, respectively (Table 6). This is the first report on cytochrome P450 activities in response to α-mangostin exposure in insects.

In this study, the activity of GST increased by a factor of 1.25 in treated larvae (Table 6). Our findings contradict the previous claims that α-mangostin inhibits GST in insects. In studies using *N. lugens,* GST activity decreased up to 3-fold after 24-h exposure to 5.44% (wt:vol) (24-h LD$_{50}$) α-mangostin (Bullangpoti et al. 2006). The differences between studies may be because of species-specific response to α-mangostin.

Esterase activities decreased in response to α-mangostin, although it had only moderate reduction of 12% ($P = 0.05$). The respective esterase activity of the control and treatment was 0.26 ± 0.01 and 0.23 ± 0.01 μmol β-napthol/mg protein/min (Table 6). These findings are consistent with the claims that α-mangostin inhibits esterase activity by 1.2-fold in *S. oryzae*

Table 6. Enzymatic activities in larvae exposed to α-mangostin

	n	Mean (SE)	P value
Cytochrome P450 activity (μmol 7-OH/mg larvae/min)			
Control	71	6.4 (0.36)	0.007
α-Mangostin treated	71	8.2 (0.6)	
Esterase activity (μmol β-napthol/ mg protein/min)			
Control	36	0.26 (0.01)	0.05
α-Mangostin treated	36	0.23 (0.01)	
GST activity (μmol/mg/min)			
Control	20	0.35 (0.02)	0.002
α-Mangostin treated	20	0.44 (0.02)	

Late third instar *Ae. aegypti* larvae (Rockefeller strain) were exposed to 2.5 μgml^{-1} of α-mangostin for 24 h. Early fourth instar larvae were used for enzyme assays. Student t tests were used to determine P value.

(Bullangpoti et al. 2004). In an attempt to determine whether α-mangostin has synergistic effects, 1 μg ml^{-1} of α-mangostin was added to six concentrations of temephos and compared with the toxicity of an equal treatment of temephos without α-mangostin. Temephos was chosen as an insecticide because it is known that esterase aids in the detoxification of organophosphates (Hemingway and Ransom 2000). Interestingly, we found that α-mangostin decreased the LC$_{50}$ of temephos by a factor of 1.6. The LC$_{50}$ of larvae exposed to α-mangostin and temephos was 0.012 (0.011–0.013) μg ml^{-1}, whereas the LC$_{50}$ of temephos alone was 0.019 (0.016–0.023) μg ml^{-1} (Table 7). Exposure of α-mangostin for 24 h at this concentration was nonlethal to third instar larvae as the mortality was <5%. Although the addition of 1 μgml^{-1} of α-mangostin decreased the LC$_{50}$ of temephos, it is not known whether these effects are synergistic or additive. It would be interesting to repeat this experiment using temephos-resistant mosquitoes, as synergistic effects may be more apparent in a resistant strain.

In summary, α-mangostin is a novel insecticide that is toxic to larvae of several mosquito species. Its novel mode of action would be a welcome addition to the limited vector control "tool box." Considering its low mammalian toxicity, it may fill a niche as an *Ae. aegypti* larvicide in storage containers for potable water. Compared with commercially available larvicides, such as temephos, α-mangostin is ≈2 orders of magnitude less toxic. However, formulations that can deliver α-mangostin to the midgut where AeSCP-2 is highly expressed may improve its efficacy. Thus, future studies should aim at delivering α-mangostin to the midgut as

Table 7. Effects of α-mangostin on temephos toxicity

Treatment	LC$_{50}$ μgml^{-1} (95% CL)	LC$_{95}$ μgml^{-1}	Slope (SE)	Hf
Temephos	0.019 (0.016–0.023)	0.041	4.8 (0.8)	2.07
Temephos + α-mangostin	0.012 (0.011–0.013)	0.023	6.2 (0.6)	0.79

Third instar *Ae. aegypti* (Rockefeller) were used. The temephos + α-mangostin treatment contained 1 μgml^{-1} of α-mangostin for 24 h (see *Materials and Methods*).

well as increasing environmental stability through improvements in formulation technology.

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